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(54) Title: METHODS FOR REGULATING BONE FORMATION

(57) Abstract

The present invention relates to methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of apoptosis of cells of osteoblastic lineage.

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TITLE OF THE INVENTION
METHODS FOR REGULATING BONE FORMATION

CROSS REFRENCE TO RELATED APPLICATIONS

The present application claims priority of U.S. provisional application Serial No. 60/104,338, filed October 15, 1998.

BRIEF DESCRIPTION OF THE INVENTION

The present invention relates to methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of apoptosis in cells of osteoblastic lineage.

BACKGROUND OF THE INVENTION

A variety of disorders in humans and other mammals involve or are associated with bone loss. Such disorders include, but are not limited to, osteoporosis, glucocorticoid induced osteoporosis, Paget's disease, abnormally increased bone turnover, periodontal disease, tooth loss, bone fractures, rheumatoid arthritis, periprosthetic osteolysis, osteogenesis imperfecta, metastatic bone disease, hypercalcemia of malignancy, and multiple myeloma. One of the most common of these disorders is osteoporosis, which in its most frequent manifestation occurs in postmenopausal women. Osteoporosis is a systemic skeletal disease characterized by a low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. Osteoporotic fractures are a major cause of morbidity and mortality in the elderly population. As many as 50% of women and a third of men will experience an osteoporotic fracture. A large segment of the older population already has low bone density and a high risk of fractures. There is a significant need to both treat and reduce the risk of (i.e. prevent) osteoporosis and other conditions associated with bone loss. Because osteoporosis, as well as other disorders associated with bone loss, are generally chronic conditions, it is believed that appropriate therapy will typically require chronic treatment.

Normal bone physiology involves a process wherein bone tissue is continuously being turned over by the processes of modeling and remodeling. In other words, there is normally an appropriate balance between resorption of existing bone tissue and the formation of new bone tissue. The exact mechanism underlying

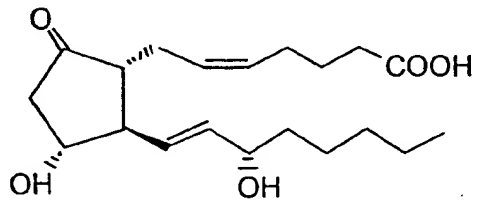
the coupling between bone resorption and formation is still unknown. However, an imbalance in these processes is manifested in various disease states and conditions of the skeleton.

Two different types of cells called osteoblasts and osteoclasts are involved in the bone formation and resorption processes, respectively. *See* H. Fleisch, *Bisphosphonates In Bone Disease, From The Laboratory To The Patient*, 3rd Edition, Parthenon Publishing (1997), which is incorporated by reference herein in its entirety.

Osteoblasts are cells that are located on the bone surface. These cells secrete an osseous organic matrix, which then calcifies. Substances such as fluoride, parathyroid hormone, and certain cytokines such as protaglandins are known to provide a stimulatory effect on osetoblast cells. However, an aim of current research is to develop therapeutic agents that will selectively increase or stimulate the bone formation activity of the osteoblasts.

Osteoclasts are usually large multinucleated cells that are situated either on the surface of the cortical or trabecular bone or within the cortical bone. The osteoclasts resorb bone in a ~~closed, sealed off~~ microenvironment located between the cell and the bone. The recruitment and activity of osteoclasts is known to be influenced by a series of cytokines and hormones. It is well known that bisphosphonates are selective inhibitors of osteoclastic bone resorption, making these compounds important therapeutic agents in the treatment or prevention of a variety of systemic or localized bone disorders caused by or associated with abnormal bone resorption. However, despite the utility of bisphosphonates, there remains the desire amongst researchers to develop additional therapeutic agents for inhibiting the bone resorption activity of osteoclasts and to increase the bone formation activity of osteoblasts.

Prostaglandins are alicyclic compounds related to the basic compound prostanoic acid. A natural prostaglandin, PGE_2, has the following structure.



Prostaglandins such as PGE_2 are known to stimulate bone formation and increase bone mass in mammals, including man. It is believed that four different receptor subtypes, designated EP_1, EP_2, EP_3, and EP_4 are involved in mediating PGE_2 effects. The major prostaglandin receptor present in bone is EP_4, which is believed to provide its effect by signaling via cyclic AMP. However, the scientific information that is currently known about the prostaglandin mediated bone process is rather limited, because the exact mechanism of action is not known. Prostaglandins and their accosted receptors are more fully described in for example, K. Ono et al., *Important role of EP_4, a subtype of prostaglandin (PG) E receptor, in osteoclast-like cell formation from mouse bone marrow cells induced by PGE_2, J. of Endocrinology,* 158, R1-R5 (1998), C.D. Funk et al., *Cloning and Expression of a cDNA for the Human Prostaglandin E Receptor EP! Subtype, Journal of Biological Chemistry*, vol. 268, no. 35, pp. 26767-26772 (1993), J.W. Reagan et al., *Cloning of a Novel Human Prostaglandin Receptor with Characteristics of the Pharmacologically Defined EP_2 Subtype, Molecular Pharmacology*, vol. 46, pp. 213-220 (1994), J. Yang et al., *Cloning and Expression of the EP_3-Subtype of Human Receptors for Prostaglandin E_2, Biochemical Biophysical Research Communication*, vol., 198, pp. 999-1006 (1994), L. Bastien et al., *Cloning, Functional Expression and Characterization of the Human Prostaglandin E_2 Receptor EP_2 Subtype, Journal Biological Chemistry*, vol. 269, pp. 11873-11877 (1994), which are all incorporated by reference herein in their entirety.

In the present invention it is found that compounds which modulate (e.g., inhibit) apoptosis, i.e. the naturally occuring process of programed cell death, in cells of osteoblastic lineage are useful for regulating bone formation. Without being limited by theory, it is believed that these compounds suppress cell death in cells of osteoblastic lineage, thereby prolonging both the activity and number of the osteoblasts.

It is an object of the present invention to provide methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

It is another object of the present invention to provide methods for treating or reducing the risk of contracting a bone related disease state or condition in a mammal in need of such treatment or prevention, comprising administering to said

mammal a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

It is another object of the present invention to provide pharmaceutical compositions useful for regulating bone formation comprising a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

It is another object of the present invention to provide pharmaceutical compositions useful for regulating bone formation comprising a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

It is another object of the present invention to identify compounds useful for inhibiting apoptosis in cells of osteoblastic lineage.

These and other objects will become readily apparent from the detailed description which follows.

SUMMARY OF THE INVENTION

The present invention relates to methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

In further embodiments, the present invention relates to methods for treating or reducing the risk of contracting a bone related disease state or condition in a mammal in need of such treatment or risk reduction, comprising administering to said mammal a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

In further embodiments, the present invention relates to methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of a compound for inhibiting apoptosis in an cells of osteoblast lineage and a bisphosphonate active.

In further embodiments, the present invention relates to pharmaceutical compositions comprising a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

In further embodiments, the present invention relates to pharmaceutical compositions comprising a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage and a bisphosphonate active.

In further embodiments, the present invention relates to the use of a composition in the manufacture of a medicament for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

All percentages and ratios used herein, unless otherwise indicated, are by weight. The invention hereof can comprise, consist of, or consist essentially of the essential as well as optional ingredients, components, and methods described herein.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage.

The periosteum, which is defined anatomically as the lining tissue of the outer cortex of long bones, plays a role in bone growth throughout life. The periosteum consists of two layers, the fibrous and cambium layer. The fibrous layer is hypocellular, consisting mainly of fibroblasts and collagenous matrix. The cambium layer consists of a thin layer of osteoblast precursors that differentiate into osteoblasts responsible for the appositional bone formation and radial growth of long bones.

Periosteal bone formation is under the control of several stimuli. Local injection onto the periosteum of Transforming growth factor (TGF) b1, bone morphogenetic protein, prostaglandin (PG)E_2, or basic fibroblast growth factor (bFGF), induce extensive periosteal bone formation. Until now, the absence of an established *in vitro* periosteal cell system has limited the understanding of the cellular responses to these and other osteogenic factors. In the present invention, a rat periosteal cell line (RP-1) is established which spontaneously immortalizes from tibia periosteal cells in culture. These RP-1 cells express the markers of the osteoblastic phenotype *in vitro*; and when placed *in vivo*, they produce a mineralized matrix. It is found that the prostaglandin PGE_2, a potent inducer of periosteal bone formation, increases RP-1 cell number *in vitro* by increasing the life span of these cells. It is found that bone metabolism, i.e. processes such as bone formation are regulated by agents that affect apoptotic cell death in bone tissue. In other words inhibitors of apoptosis of cells of osteoblastic lineage are found useful for regulating bone formation.

It is found in the present invention that RP-1 cells display the characteristics of osteoblast precursors that are able to differentiate in culture, express osteoblastic markers at confluence, and mineralize extracellular matrix. The ability of RP-1 cells to form mineralized matrix *in vivo* is determined in nude mice. It is found that six to eight weeks after inoculation of RP-1 cells, palpable tumors are detected at the site of injection. To confirm that the tumors contain bone tissue, paraffin sections are analyzed for mineral content and the expression of alkaline phosphatase (ALP) and osteocalcin (OC). It is seen that RP-1 cells are able to differentiate, express the osteoblast phenotypic markers, and form a calcified matrix *in vivo* in nude mice.

The prostaglandin E_2 increases RP-1 periosteal cell number by suppressing apoptosis. PGE_2 is knwon to be a potent inducer of periosteal bone formation *in vivo* in rats and humans. In the present invention, the effect of PGE_2 on RP-1 periosteal cells is demonstrated. It is seen that treatment of RP-1 cells with PGE_2 (0.01microM - 1microM) in the presence of 2% serum for five days, increases cell number in a dose-dependent manner with a maximum effect at 1microM. The increase in cell number could result from an increase in proliferation and /or life span of these cells. However, it is seen that PGE_2 has no significant effect on [^{3}H]thymidine incorporation in RP-1 cells, indicating that the increased cell number is not a result of increased proliferation.

The effect of PGE_2 on RP-1 cell survival is examined. Quantitative analysis of DNA content by flow cytometry shows that, in the presence of 2% FBS, 15% of RP-1 cells undergo apoptosis after 48 hours of culture. PGE_2 (1 μM) suppresses apoptosis by 48%. Suppression of apoptosis in RP-1 by PGE_2 is seen *in situ* using Transferase-mediated digoxygenin-dUTP Nick End-Labeling, hereafter "TUNEL". PGE_2 substantially decreases the number of positively stained nuclei that contain fragmented DNA. PGE_2 is seen to ncrease PR-1 cell number by suppressing apoptosis.

Previously, limited information has been available regarding the occurrence of apoptosis in adult bone tissue. In the present invention, paraffin sections of adult rat tibia aere subjected to TUNEL staining showing positively stained nuclei at various sites of bone such as the periosteum, endosteum, trabecular bone surface, and bone marrow. It is seen that the occurrence of apoptosis in bone cells shows that the life span of these cells can be regulated by osteotrophic factors, such as PGE_2.

In the present invention it is found that RP-1 cells, spontaneously immortalized from primary periosteal cell culture derived from adult rat tibia, express high levels of COL-I mRNA in early culture, followed by the expression of ALP and OC mRNAs, and finally by nodule formation and mineralization. The sequential expression of osteoblast phenotypic markers in RP-1 cells is similar to that seen during the differentiation of calvaria, bone marrow, and chick tibia periosteum-derived primary cell cultures. *In vivo* inoculation of RP-1 cells produces tumors that contain a mineralized matrix. Alkaline phosphatase and osteocalcin stainings show a differential distribution of these two osteoblastic markers, OC being associated with cells at the mineralized foci in the center of the tumor, and ALP being localized in peripheral cells.

Although periosteal bone formation *in vivo* is regulated by various humoral and physical stimuli, their mechanisms of action are unknown. *In vivo,* PGE_2 stimulates bone formation by increasing the number of active osteoblasts on the bone surface. This may occur by stimulating the recruitment of new osteoblast precursors and/or prolonging the life span of existing osteoblasts on the bone surface. It is found in the present invention that PGE_2 increases RP-1 periosteal cell number *in vitro* by suppressing apoptosis, without affecting proliferation. Similar effects are also observed using PGE_1 and forskolin. Without being limited by theory, it is believed that cyclic AMP is mediating the process.

Prostaglandins E (especially PGE_2) stimulate bone formation and increase bone mass in several species, including man. The mechanism of this effect, the target cells and the receptors involved are not completely known. Specific cell-surface receptors for PGE_2, such as EP_{1-4}, which employ different secondary messenger systems have been cloned and characterized. It is believed that cyclic AMP may have a role in osteogenesis induced by PGE_2. The expression of the EP_2 and EP_4 receptors that are coupled to the cAMP pathway is investigated in the bone tissue of young adult rats (where PGE_2 is markedly anabolic), and in various osteoblastic cell lines. Osteoblastic cell lines, RCT-1, RCT-3, TRAB-11 and RP-1, as well as osteoblastic cells harvested from fetal rat bones express EP_4 mRNA but not EP_2 mRNA. In addition, EP_4 mRNA is expressed in tibiae and calvariae of 5-week-old rats while EP_2 is not. Treatment of periosteal cells (RP-1) *in vitro* with 10^{-6} M PGE_2 increases the level of EP_4 mRNA which peaks at 2 hours. Similarly, systemic administration of an anabolic dose of PGE_2 (3-6 mg/kg) to young adult rats

upregulates the expression of EP_4 in tibiae and calvariae, an effect which peaks at 1-2 hours. Using in-situ hybridization it is found that the increased expression of EP_4 mRNA in the tibial metaphysis following systemic PGE_2 treatment is localized to bone marrow cells.

EP_4 is expressed in osteoblastic cells *in vitro* and in bone marrow putative osteoprogenitor cells *in vivo* and is upregulated by its ligand, PGE_2. Given the presence of EP_4 expression in the cells examined and in bone tissue, it is believed that EP_4 is the receptor subtype which mediates the anabolic effects of PGE_2.

Prostaglandins (especially PGE_2) have multiple effects on bone, stimulating both resorption and formation. Systemic administration of PGE_2 or E_1 to infants and to animals is clearly anabolic, stimulating bone formation and increasing bone mass. Also local administration of PGE_2 into long bones stimulates new bone formation, suggesting that that PGE_2 acts directly on bone tissue to induce osteogenesis. Histological analysis of bones treated with PGE_2 indicates that PGE_2 increases the number of osteoblasts present on the bone surface, suggesting that prostaglandins act by recruiting osteoblasts from their precursors.

PGEs act on various cells via specific cell-surface receptors divided into 4 subtypes, EP_{1-4}, according to their relative sensitivity to selective agonists and antagonists. The receptor subtypes belong to the family of G-protein-coupled receptors and activate distinct secondary messenger systems such as adenylate cyclase or phospholipase C. Of these 4 receptors, EP_4 and EP_2 activate adenylate cyclase, EP_1 activates phospholipase C, and EP_3 either lowers intracellular cAMP levels or activates phospholipase C, depending on the specific spliced variant.

In osteoblastic cells *in vitro*, PGE_2 stimulates both phosphatidylinositol and cyclic AMP transduction pathways. Both EP_1 and EP_4, found in osteoblastic MC3T3-E_1 cells play a role in the biological action of PGE_2 in bone tissue. Also PGE_1, a potent inducer of bone formation in humans and other species, increases intracellular cyclic AMP but has no effect on phosphatidylinositol turnover in osteoblastic cells. Without being limited by theory, it is therefore believed that PGE receptors coupled to adenylate cyclases, EP_2 and/or EP_4, are involved in osteogenesis. Initial characterization of *in vivo* expression of EP receptors by in situ hybridization shows that in embryonic and neonatal mice EP_4 is the major form found in bone tissue, especially in preosteoblasts. See Ikeda T, Miyaura C, Ichikawa A, Narumiya S, Yoshiki S and Suda T 1995, *In situ localization of three subtypes*

(EP_1, EP_3 and EP_4) of prostaglandin E receptors in embryonic and newborn mice. J Bone Miner Res **10** (sup 1):S172, and R.S. Weinstein et al., *Inhibition of osteoblastogenesis and promotion of apoptosis of osteoblasts and osteocytes by glucocorticoids -- Potential mechanisms of their deleterious effects on bone, J. of Clinical of Investigation*, 102 (2), pp. 274-282 (1998), which are incorporated by reference herein in its entirety.

Also, it is found that EP_4 but not EP_2 mRNA is expressed in adult rat bone tissue and bone-derived cell lines and that expression is stimulated by PGE_2.

Analysis of the *in vivo* expression of PGE receptors shows that EP_4 but not EP_2 is expressed in total RNA from adult rat tibiae and calvariae. EP_4 is believed to be the major adenylate cyclase-coupled PGE_2 receptor expressed in osteoblastic cells and in bone tissue. Also, the EP_4 receptor subtype is expressed in the bone tissue of young adult rats, in which PGE_2 is strongly anabolic.

EP_4 mRNA is expressed in osteoblast precursor cells. It is also found in less differentiated bone cell lines such as RCT-1, TRAB-11 and the RP-1 periosteal cells, but not in fibroblasts. It is highly expressed in bone marrow cells that include osteoblast precursor cells, but not in fully mature osteoblasts on the bone surface. It is believed that PGE_2 induces osteogenesis via an increase in the number of active osteoblasts present on the bone surface, resulting from the recruitment of osteoblast precursor cells rather than the enhancement of the activity of existing osteoblasts.

It is found that osteoblast precursors are the major target cells for the anabolic effect of PGE_2 and that its action in these cells is mediated by EP_4. The EP_4 receptor subtype is believed to be the major receptor which mediates the effects of PGE_2 in rat bone tissue. Induction of EP_4 by PGE_2 further supports its biological role in the bone tissue and points to a mechanism of autoamplification of PGE action.

Despite the scientific information that is known on prostaglandins and protaglandin receptor subtypes, it has previously neither been taught nor suggested that inhibitors of apoptosis of cells of osteoblastic lineage would be useful for regulating bone formation.

Methods Of Regulating Bone Formation

The present invention relates to methods for regulating bone formation in a mammal comprising administering to a mammal in need thereof a therapeutically effective amount of an inhibitor of apoptosis in cells of osteoblastic lineage. By

"regulating bone formation" as used herein means that that biochemical processes involved in bone formation, i.e. bone building or osteogenesis, are altered, modified, or effected by administration of the therapeutic agents of the present invention. "Regulating bone formation" is also intended to include stimulating bone formation.

By "cells of osteoblastic lineage", as used herein, is meant cells that include osteoblasts, osteocytes, and bone lining cells, and all other cells that can differentiate and give rise to these cells. In other words, the term is intended to include all such related cells that are involved in bone formation (i.e. osteogenesis). The term "cells of osteoblastic lineage" is intended to specifically exclude bone resorbing cells such as osteoclasts and other related cells that can differentiate and give rise to osteoclasts. A nonlimiting example of a cell of osteoblastic lineage is an RP-1 cell.

The methods and compositions of the present invention are useful for both treating and reducing the risk of disease states or conditions associated with abnormal bone metabolism. Such disease states or conditions include, but are not limited to, osteoporosis, glucocorticoid induced osteoporosis, Paget's disease, abnormally increased bone turnover, periodontal disease, tooth loss, bone fractures, rheumatoid arthritis, periprosthetic osteolysis, osteogenesis imperfecta, metastatic bone disease, hypercalcemia of malignancy, and multiple myeloma.

In further embodiments, the methods comprise administering a therapeutically effective amount of a compound for inhibiting apoptosis in cells of osteoblastic lineage and a bisphosphonate active. Both concurrent and sequential administration of the inhibitor and bisphosphonate are deemed within the scope of the present invention. With sequential administration, the inhibitor and the bisphosphonate can be administered in either order. In a subclass of sequential administration the inhibitor and bisphosphonate are typically administered within the same 24 hour period. In yet a further subclass, the inhibitor and bisphosphonate typically administered within about 4 hours of each other.

The term "therapeutically effective amount", as used herein, means that amount of the inhibitor of apoptosis of cells of osteoblastic lineage, or other actives of the present invention, that will elicit the desired therapeutic effect or response or provide the desired benefit when administered in accordance with the desired treatment regimen. A prefered therapeutically effective amount is a bone formation regulating amount.

"Pharmaceutically acceptable" as used herein, means generally suitable for administration to a mammal, including humans, from a toxicity or safety standpoint.

In the present invention, the inhibitor is typically administered for a sufficient period of time until the desired therapeutic effect is achieved. The term "until the desired therapeutic effect is achieved", as used herein, means that the therapeutic agent or agents are continuously administered, according to the dosing schedule chosen, up to the time that the clinical or medical effect sought for the disease or condition being mediated is observed by the clinician or researcher. For methods of treatment of the present invention, the compounds are continuously administered until the desired change in bone mass or structure is observed. In such instances, achieving an increase in bone mass or a replacement of abnormal bone structure with normal bone structure are the desired objectives. For methods of reducing the risk of a disease state or condition, the compounds are continuously administered for as long as necessary to prevent the undesired condition. In such instances, maintenance of bone mineral density is often the objective.

Nonlimiting examples of administration periods can range from about 2 weeks to the remaining lifespan of the mammal. For humans, administration periods can range from about 2 weeks to the remaining lifespan of the human, preferably from about 2 weeks to about 20 years, more preferably from about 1 month to about 20 years, more preferably from about 6 months to about 10 years, and most preferably from about 1 year to about 10 years.

Methods Of Identifying Inhibitors Of Apoptosis of Cells of Osteoblastic Lineage

The present invention also relates to methods for identifying compounds useful as inhibitors of apoptosis of cells of osteoblastic lineage. Compounds so identified are useful for stimulating bone formation.

The present invention relates to a method for identifying compounds which inhibit apoptosis of cells of osteoblastic lineage comprising:

a). contacting a putative inhibitor of apoptosis of cells of osteoblastic lineage with a cell culture; and

b). determining the inhibition by comparing the cell culture so-contacted (i.e. the cell culture contacted with said putative inhibitor) with a cell culture not contacted with said putative inhibitor.

Compositions Of The Present Invention

The pharmaceutical compositions of the present invention comprise a therapeutically effective amount of an inhibitor of apoptosis of cells of osteoblastic lineage.

These compositions can further comprise a pharmaceutically-acceptable carrier. In further embodiments these compositions also comprise a bisphosphonate active.

Inhibitors Of Apoptosis Of Cells of Osteoblastic Lineage

The methods and compositions of the present invention comprise an inhibitor of apoptosis of cells of osteoblastic lineage.

Apoptosis is the normal process of programmed cell death, either *in vitro* or in an organism.

The inhibitors of the present invention are found to inhibit or suppress apoptosis of cells of osteoblastic lineage. These compounds are found to have an EC_{50} value of from about 0.1 nanoM to about 10 microM. In a further subclass of the present invention these compounds have an EC_{50} value from about 0.01 microM to about 1 microM. In a yet further subclass of the the present invention these compounds have an EC_{50} value from about 0.1 microM to about 1 microM.

EC_{50} is a parameter familiar to one skilled in the art and is defined as the concentration or dose of an inhibitor that is needed to produce a 50% reduction of a maximum response. See also, Goodman and Gilman's, *The Pharmacologic Basis of Therapeutics*, 9th edition, 1996, chapter 2, E. M. Ross, *Pharmacodynamics, Mechanisms of Drug Action and the Relationship Between Drug Concentration and Effect*, which is incoroporated by reference herein in its entirety.

Nonlimiting examples of inhibitors are selected from the group consisting of prostaglandin E_1, prostaglandin E_2, forskolin (7β-acetoxy-8,13-epoxy-1α,6β,9α-trihydroxylabd-14-3-en-11-one), caspase inhibitors, and mixtures thereof.

The caspase inhibitors useful herein are generally relatively short aspartic acid-containing peptides, although non-peptide inhibitors are also intended as being within the scope of the present invention. By "relatively short", as used herein means that the peptides typically contain from about 3 to about 5 amino acids in length. By "aspartic acid-containing" is meant that these peptides comprise at least one aspartic acid moiety, preferably at the carboxy-terminal end. These peptides are preferably blocked at both the amino and carboxy terminal ends with blocking groups.

The caspase inhibitors useful herein can be represented by the following chemical formula

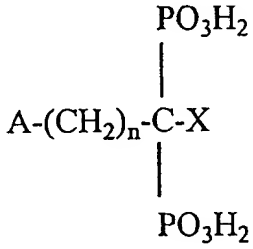
$$(ATBG) - (AA)_n - (Asp) - (CTBG)$$

wherein (ATBG) is an amino terminal blocking group, (AA) is an amino acid moiety, "Asp" is aspartic acid moiety, (CTBG) is a carboxy terminal blocking group, and n is an integer from about 2 to about 4. In the caspase inhibitors, the amino acid (AA) can be selected from any of the naturally occurring amino acids, the D-enantiomers of the naturally-occurring amino acids (for example, D-alanine), and non-naturally occurring amino acids (for example, 3-aminopropionic acid and N-methyl glycine). The (ATBG) and (CTBG) moities are selected from any of the blocking groups that are well known to peptide chemists of ordinary skill in the art. *See* Greene, T.W. et al., Protecting Groups in Organic Synthesis, 2nd edition, 1991, John Wiley & Sons, Inc., which is incorporated by reference herein in its entirey. Nonlimiting examples of (ATBG) moieties are bezyloxycarbonyl group (also known as the cbz or Z group) and the *t*-butoxycarbonyl group (also known as the boc group), and the acyl group. Nonlimiting examples of (CTBG) moieties are alkyl groups (for example methyl and ethyl esters), the benzyl group, and the fluoromethyl keto group [which is abbreviated as (OMe)-CH_2F or FMK].

Nonlimiting examples of caspase inhibitors useful herein are disclosed in U.S. Patent No. 5,210, 272, to Palmer, issued May 11, 1993, U.S. Patent 5,101,068, to Palmer et al., issued March 31, 1992, and U.S. Patent No. 4,518,528, to Rasnick, issued May 21, 1985, which are all incorporated by reference herein in their entirety. Preferred caspase inhibitors useful herein are selected from the group consisting of Z-Val-Ala-Asp-FMK (which has a molecular weight of about 468), Z-Asp-Glu-Val-Asp-FMK (which has a molecular weight of about 668), and Z-Tyr-Val- Ala-Asp-FMK (which has a molecular weight of about 630) MW 630. In the foregoing the standard three-letter amino acid abbreviations are used.

Bisphosphonates

The methods and compositions of the present invention, can further comprise a bisphosphonate active. The bisphosphonates of the present invention correspond to the chemical formula



wherein n is an integer from 0 to 7 and wherein A and X are independently selected from the group consisting of H, OH, halogen, NH_2, SH, phenyl, C1-C30 alkyl, C3-C30 branched or cycloalkyl, C1-C30 substituted alkyl, C1-C10 alkyl substituted NH_2, C3-C10 branched or cycloalkyl substituted NH_2, C1-C10 dialkyl substituted NH_2, C3-C10 branched or cycloalkyl disubstituted NH_2, C1-C10 alkoxy, C1-C10 alkyl substituted thio, thiophenyl, halophenylthio, C1-C10 alkyl substituted phenyl, pyridyl, furanyl, pyrrolidinyl, imidazolyl, imidazopyridinyl, and benzyl, such that both A and X are not selected from H or OH when n is 0; or A and X are taken together with the carbon atom or atoms to which they are attached to form a C3-C10 ring.

In the foregoing chemical formula, the alkyl groups can be straight, branched, or cyclic, provided that sufficient atoms are selected for the chemical formula. The C1-C30 substituted alkyl can include a wide variety of substituents, nonlimiting examples which include those selected from the group consisting of phenyl, pyridyl, furanyl, pyrrolidinyl, imidazonyl, NH_2, C1-C10 alkyl or dialkyl substituted NH_2, OH, SH, and C1-C10 alkoxy.

The foregoing chemical formula is also intended to encompass complex carbocyclic, aromatic and hetero atom structures for the A and/or X substituents, nonlimiting examples of which include naphthyl, quinolyl, isoquinolyl, adamantyl, and chlorophenylthio.

A non-limiting class of structures useful in the instant invention are those in which A is selected from the group consisting of H, OH, and halogen, X is selected from the group consisting of C1-C30 alkyl, C1-C30 substituted alkyl, halogen, and C1-C10 alkyl or phenyl substituted thio, and n is 0.

A non-limiting subclass of structures useful in the instant invention are those in which A is selected from the group consisting of H, OH, and Cl, X is selected from the group consisting of C1-C30 alkyl, C1-C30 substituted alkyl, Cl, and chlorophenylthio, and n is 0.

A non-limiting example of the subclass of structures useful in the instant invention is when A is OH and X is a 3-aminopropyl moiety, and n is 0, so that the resulting compound is a 4-amino-1,-hydroxybutylidene-1,1-bisphosphonate, i.e. alendronate.

Pharmaceutically acceptable salts and derivatives of the bisphosphonates are also useful herein. Nonlimiting examples of salts include those selected from the group consisting alkali metal, alkaline metal, ammonium, and mono-, di, tri-, or tetra-C1-C30-alkyl-substituted ammonium. Preferred salts are those selected from the group consisting of sodium, potassium, calcium, magnesium, and ammonium salts. Nonlimiting examples of derivatives include those selected from the group consisting of esters, hydrates, and amides.

It should be noted that the terms "bisphosphonate" and "bisphosphonates", as used herein in referring to the therapeutic agents of the present invention are meant to also encompass diphosphonates, biphosphonic acids, and diphosphonic acids, as well as salts and derivatives of these materials. The use of a specific nomenclature in referring to the bisphosphonate or bisphosphonates is not meant to limit the scope of the present invention, unless specifically indicated. Because of the mixed nomenclature currently in use by those or ordinary skill in the art, reference to a specific weight or percentage of a bisphosphonate compound in the present invention is on an acid active weight basis, unless indicated otherwise herein. For example, the phrase "about 5 mg of a bisphosphonate selected from the group consisting of alendronate, pharmaceutically acceptable salts thereof, and mixtures thereof, on an alendronic acid active weight basis" means that the amount of the bisphosphonate compound selected is calculated based on 5 mg of alendronic acid. For other bisphosphonates, the amount of bisphosphonate is calculated based on the corresponding bisphosphonic acid.

Nonlimiting examples of bisphosphonates useful herein include the following:

Alendronic acid, 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid.

Alendronate (also known as alendronate sodium or alendronate monosodium trihydrate), 4-amino-1-hydroxybutylidene-1,1-bisphosphonic acid monosodium trihydrate.

Alendronic acid and alendronate are described in U.S. Patents 4,922,007, to Kieczykowski et al., issued May 1, 1990; 5,019,651, to

Kieczykowski et al., issued May 28, 1991; 5,510,517, to Dauer et al., issued April 23, 1996; 5,648,491, to Dauer et al., issued July 15, 1997, all of which are incorporated by reference herein in their entirety.

Cycloheptylaminomethylene-1,1-bisphosphonic acid, YM 175, Yamanouchi (cimadronate), as described in U.S. Patent 4,970,335, to Isomura et al., issued November 13, 1990, which is incorporated by reference herein in its entirety.

1,1-dichloromethylene-1,1-diphosphonic acid (clodronic acid), and the disodium salt (clodronate, Procter and Gamble), are described in Belgium Patent 672,205 (1966) and *J. Org. Chem 32*, 4111 (1967), both of which are incorporated by reference herein in their entirety.

1-hydroxy-3-(1-pyrrolidinyl)-propylidene-1,1-bisphosphonic acid (EB-1053).

1-hydroxyethane-1,1-diphosphonic acid (etidronic acid).

1-hydroxy-3-(N-methyl-N-pentylamino)propylidene-1,1-bisphosphonic acid, also known as BM-210955, Boehringer-Mannheim (ibandronate), is described in U.S. Patent No. 4,927,814, issued May 22, 1990, which is incorporated by reference herein in its entirety.

6-amino-1-hydroxyhexylidene-1,1-bisphosphonic acid (neridronate).

3-(dimethylamino)-1-hydroxypropylidene-1,1-bisphosphonic acid (olpadronate).

3-amino-1-hydroxypropylidene-1,1-bisphosphonic acid (pamidronate).

[2-(2-pyridinyl)ethylidene]-1,1-bisphosphonic acid (piridronate) is described in U.S. Patent No. 4,761,406, which is incorporated by reference in its entirety.

1-hydroxy-2-(3-pyridinyl)-ethylidene-1,1-bisphosphonic acid (risedronate).

(4-chlorophenyl)thiomethane-1,1-disphosphonic acid (tiludronate) as described in U.S. Patent 4,876,248, to Breliere et al., October 24, 1989, which is incorporated by reference herein in its entirety.

1-hydroxy-2-(1^{H}-imidazol-1-yl)ethylidene-1,1-bisphosphonic acid (zolendronate).

A non-limiting class of bisphosphonates useful in the instant invention are selected from the group consisting of alendronate, cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

A non-limiting subclass of the above-mentioned class in the instant case is selected from the group consisting of alendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

A non-limiting example of the subclass is alendronate monosodium trihydrate.

Other Components Of The Pharmaceutical Compositions

The inhibitors of apoptosis of cells of osteoblastic lineage, and in further embodiments the bisphosphonate actives and any other additional actives, are typically administered in admixture with suitable pharmaceutically acceptable diluents, excipients, or carriers, collectively referred to herein as "carrier materials", suitably selected with respect to the mode of administration. Nonlimiting examples of product forms include tablets, capsules, elixirs, syrups, powders, suppositories, nasal sprays, liquids for ocular administration, formulations for transdermal administration, and the like, consistent with conventional pharmaceutical practices. For example, for oral administration in the form of a tablet, capsule, or powder, the active ingredient can be combined with an oral, non-toxic, pharmaceutically acceptable inert carrier such as lactose, starch, sucrose, glucose, methyl cellulose, magnesium stearate, mannitol, sorbitol, croscarmellose sodium and the like. For oral administration in liquid form, e.g., elixirs and syrups, the oral drug components can be combined with any oral, non-toxic, pharmaceutically acceptable inert carrier such as ethanol, glycerol, water and the like. Moreover, when desired or necessary, suitable binders, lubricants, disintegrating agents and coloring agents can also be incorporated. Suitable binders can include starch, gelatin, natural sugars such a glucose, anhydrous lactose, free-flow lactose, beta-lactose, and corn sweeteners, natural and synthetic gums, such as acacia, guar, tragacanth or sodium alginate, carboxymethyl cellulose, polyethylene glycol, waxes, and the like. Lubricants used in these dosage forms include sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride and the like. An example of a tablet formulation is that described in U.S. Patent No. 5,358,941, to Bechard et al, issued October 25,

1994, which is incorporated by reference herein in its entirety. The compounds used in the present method can also be coupled with soluble polymers as targetable drug carriers. Such polymers can include polyvinylpyrrolidone, pyran copolymer, polyhydroxylpropyl-methacrylamide, and the like.

The following Examples are presented to better illustrate the invention.

EXAMPLES

1. Periosteal Cell Isolation And Immortalization

Periostea are dissected from the antero-medial surface of tibia of 2 - 3 week-old Sprague Dawley rats (Taconic, Germantown, NY). The periostea collected are minced into small pieces, and are subjected to collagenase (1mg/ml) digestion for 1 hour at 37°C, to release periosteal cells. Primary periosteal cells are cultured in the presence of DMEM supplemented with 10% FBS and are subcultured every week. Colonies of cells which form after six weeks of culture, are isolated using cloning cylinders, and are sub-cloned by limiting dilution. RP-1 clonal periosteal cell line is selected and characterized. See A. Grigoriadis et al, *In vitro differentiation of bone and hypertrophic cartilage from periosteal-derived cells, Differentiation*, 60, 229-307 (1996) and H. Nakahara et al., *Bone and cartilage formation in diffusion chambers by subcultured cells derived from the periosteum, Bone* , 11, 181-188 (1990), which are both incorporated by reference herein in their entirety.

2. Cell Culture And Stainings

RP-1 cells are cultured in DMEM supplemented with 10% fetal bovine serum. For the determination of PGE_2 effect on cell number, RP-1 cells are plated at 100,000 cells per well in 24-well plates (Costar, Cambridge, MA) and cultured for 24 hours. Cells are cultured in presence or absence of PGE_2 (10^{-7} - 10^{-8} M) in media supplemented with 2% serum. After 5 days, cells are trypsinized and counted using a coulter counter (Coulter Electronics LTD, Lutton, England).

For ALP staining, RP-1 cells are plated at about 20,000 cells per well in 24-well plates and cultured in DMEM supplemented with 10% serum for six days.

Cells are washed in calcium free PBS, fixed, and stained for ALP using naphtol ASBI as substrate.

For nodule formation, RP-1 cells are plated at about 5,000 cells per well in 24-well plates and cultured for two weeks in DMEM supplemented with 10% serum. 10mM β-glycerophosphate is added two days before ending the culture. Cells are washed in calcium free PBS, fixed, and stained with Von Kossa.

3. Subcutaneous Injection Of RP-1 In Nude Mice

About five million RP-1 cells suspended in 100ml DMEM without serum are subcutaneously injected in eight week-old male nude mice (Taconic, Germantown, NY), maintained under aseptic conditions. Three out of five mice develop tumors at the site of injection, after six to eight weeks. The tumors are harvested, fixed in 4% paraformaldehyde, and processed for paraffin sectioning. Deparaffinized sections aere stained with toluidine blue, ALP, and Von Kossa.

4. RNA Isolation And Northern Blot Analysis

RP-1 periosteal cells are plated at about 10,000 cells/cm^2 in 100-mm culture plates (Costar, Cambridge, MA) and cultured for 3, 6 and 10 days. Total RNA is extracted. See P. Chomczynski et al., *Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, Anal. Biochem.*, 162, 156-159 (1987), which is incorporated by reference herein in its entirety. RNA (15 microg) is electrophoresed through 1% agarose-formaldehyde and blotted onto a nylon membrane (Hybond N, Amersham). The filters are prehybridized in buffer containing 50% formamide, 5 x SSC (1 x SSC = 0.15 M NaCl/0.015 M sodium citrate), 5 x Denhardt's solution and salmon sperm DNA (100 mg/ml), and hybridized at 42°C in fresh buffer containing mouse type I collagen (COL-I) a1, rat alkaline phosphatase (ALP), or rat osteocalcin (OC) cDNA probes labeled with [a-^{32}P]deoxy-CTP using a random primer DNA-labeling kit.

5. Immunohistochemistry

Deparaffinized sections of RP-1 tumors and fixed RP-1 cells are first permeabilized with 0.1% TritonX100 (in PBS) for about 30 min. The slides are then treated with 2%BSA for about 30 min to block non specific binding, and with 0.5% hydrogen peroxide for about 30 min to quench endogenous peroxidases. The slides

are washed three times in 0.1% TritonX100 (in PBS) for about 10 min each. This step is repeated after each of the subsequent steps. Sections are exposed to a rabbit anti-rat OC anti-serum, diluted 1 to 200 in a solution containing 0.1% BSA and 0.1% tritonX100. Non immune rabbit serum is used as a negative control. The samples are exposed to a horseradish peroxidase conjugated secondary antibody (Amersham, Arlington Heights, IL), and developed with diaminobenzidine. Sections are counterstained with methyl green and visualized under a light microscope.

6. [^{3}H] Thymidine Incorporation

RP-1 cells are plated in 24-well plates (Costar, Cambridge, MA) at about 50,000 cells per well and cultured for 24 hours. Cells are cultured without serum for an additional 24 hours, and are treated with PGE_2 (1mM) in the presence of 2% FBS for 20 hours. [^{3}H]thymidine (0.1 mci/ml) is added 2 hours prior to culture arrest, and incorporated thymidine is determined as previously described. See S. Rodan et al., *Growth stimulation of rat calvaria osteoblastic cells by acidic fibroblast growth factor, Endocrinology.* 121, 1917-1923 (1987), which is incorporated by reference herein in its entirety.

7. Flow Cytometry

RP-1 cells are plated at 50,000 cells/cm^2 in 24-well plates (Costar, Cambridge, MA) and are cultured for 2 days in DMEM supplemented with 10% FBS. Cells are treated with PGE_2 (1mM) for 48 hours in DMEM supplemented with 2% FBS. For analysis of DNA content by flow cytometry, the cells are trypsinized and single cell suspensions are prepared. The cells are washed twice in Ca^{2+} Mg^{2+}-free PBS and fixed in ethanol/PBS (3 : 1 vol/vol) for 30 min. After centrifugation, the cells are washed in PBS, incubated with 0.5 mg/ml RNAse A, and stained with propidium iodide at a final concentration of 50 mg/ml. DNA content and cell cycle profile are analyzed with a FACScan flow cytometer (Becton Dickinson, San Francisco, CA). The red fluorescence is excited at 488 nm wave length of the Argon laser beam. The data acquisition and analysis are performed using cellQuest and ModFit software (Becton Dickinson, San Francisco, CA), respectively.

8. TUNEL Assay

For *in situ* identification of apoptosis, a TUNEL assay is used, according to the manufacturer's recommendations (Oncor, Gaithersburg, MD). Paraffin sections of four week-old rat tibia are deparaffinized and exposed to proteinase K (10 mg/ml in PBS) for 15 min. RP-1 cells and tibia sections are incubated in 0.5% H_2O_2 in PBS for 15 min, to quench endogenous peroxidases. After washing in PBS, the samples are incubated with nucleotide terminal transferase in the presence of dioxygenin-11-dUTP. Labeled cells are identified using an anti-dioxygenin HRP-conjugated antibody. As a control, the samples are exposed to the same mixture excluding the terminal transferase. The samples are counterstained with methyl green and visualized under a light microscope.

9. Other Inhibitors Of Apoptosis Of RP-1 Cells

Using the methods of the present invention as described in Examples 1-8, in addition to prostaglandin E_2, it is found that prostaglandin E_1, forskolin, and caspase inhibitors, are effective for inhibiting apoptosis of cells of osteoblastic lineage.

10. Pharmaceutial tablets

Pharmaceutical tablets are prepared using standard mixing and formation techniques.

Tablets containing about 1 to 100 mg of an inhibitor of cells of osteoblastic lineage are prepared using the following relative weights of ingredients.

Ingredient	Per Tablet
Inhibitor of the present invention	1 to 100 mg
Anhydrous Lactose, NF	71.32 mg
Magnesium Stearate, NF	1.0 mg
Croscarmellose Sodium, NF	2.0 mg
Microcrystalline Cellulose, NF	QS 200 mg

The resulting tablets are useful for administration in accordance with the methods of the present invention for inhibiting apoptosis of cells of osteoblastic lineage and for regulating bone resorption.

In further embodiments, tablets are prepared that also contain 5 or 10 mg of a bisphosphonate active, on a bisphosphonic acid active basis, of a bisphosphonate selected from the group consisting of alendronate cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, and pharmaceutically acceptable salts thereof.

11. Liquid Formulation.

Liquid formulations are prepared using standard mixing techniques.

A liquid formulation containing about 1 to about 100 mg of an inhibitor of cells of osteoblastic lineage is prepared using the following relative weights of ingredients.

Ingredient	Weight
Inhibitor of the present invention	1-100 mg
Sodium Propylparaben	22.5 mg
Sodium Butylparaben	7.5 mg
Sodium Citrate Dihydrate	1500 mg
Citric Acid Anhydrous	56.25 mg
Sodium Saccharin	7.5 mg
Water	qs 75 mL
1 N Sodium Hydroxide (aq)	qs pH 6.75

The resulting liquid formulation is useful for administration for inhibiting apoptosis of cells of osteoblastic lineage and for regulating bone formation.

In further embodiments solutions are prepared also containing 5 or 10 mg of a bisphosphonate active, on a bisphosphonic acid active basis, of a bisphosphonate selected from the group consisting of alendronate cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, and pharmaceutically acceptable salts thereof.

WHAT IS CLAIMED IS:

1. A method for regulating bone formation in a mammal in need thereof comprising administering to said mammal a therapeutically effective amount of an inhibitor of apoptosis of cells of osteoblastic lineage.

2. A method according to Claim 1 wherein said mammal is a human.

3. A method for treating or reducing the risk of contracting a disease state or condition in a mammal in need of such treatment or risk reduction, comprising administering to said mammal a therapeutically effective amount of an inhibitor of apoptosis of cells of osteoblastic lineage.

4. A method according to Claim 3 wherein said mammal is a human.

5. A method according to Claim 4 wherein said disease state or condition is selected from the group consisting of osteoporosis, glucocorticoid induced osteoporosis, Paget's disease, abnormally increased bone turnover, periodontal disease, tooth loss, bone fractures, rheumatoid arthritis, periprosthetic osteolysis, osteogenesis imperfecta, metastatic bone disease, hypercalcemia of malignancy, and multiple myeloma.

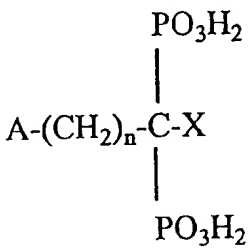
6. A method according to Claim 5 wherein said disease state or condition is selected from the group consisting of osteoporosis, glucocorticoid induced osteroporosis, and Paget's disease.

7. A method according to Claim 1 wherein said inhibitor is selected from the group consisting of PGE_1, PGE_2, forskolin, caspase inhibitors, and mixtures thereof.

8. A method for regulating bone formation in a mammal in need thereof comprising administering to said mammal a therapeutically effective amount

of an inhibitor of apoptosis of cells of osteoblastic lineage and a bisphosphonate active.

9. A method according to Claim 8 wherein said bisphosphonate active corresponds to the chemical structure



wherein n is an integer from 0 to 7 and wherein A and X are independently selected from the group consisting of H, OH, halogen, NH_2, SH, phenyl, C1-C30 alkyl, C3-C30 branched or cycloalkyl, C1-C30 substituted alkyl, C1-C10 alkyl substituted NH_2, C3-C10 branched or cycloalkyl substituted NH_2, C1-C10 dialkyl substituted NH_2, C1-C10 alkoxy, C1-C10 alkyl substituted thio, thiophenyl, halophenylthio, C1-C10 alkyl substituted phenyl, pyridyl, furanyl, pyrrolidinyl, imidazolyl, imidazopyridinyl, and benzyl; or A and X are taken together with the carbon atom or atoms to which they are attached to form a C3-C10 ring; and provided that when n is 0, A and X are not selected from the group consisting of H and OH; and the pharmaceutically acceptable salts thereof.

10. A method according to Claim 8 wherein said bisphosphonate active is selected from the group consisting of alendronate, cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

11. A method according to Claim 10 wherein said bisphosphonate active is alendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

12. A method according to Claim 11 wherein said bisphosphonate active is alendronate monosodium trihydrate.

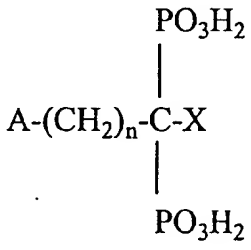
13. A pharmaceutical composition comprising a therapeutically effective amount of an inhibitor of apoptosis of cells of osteoblastic lineage.

14. A pharmaceutical composition according to Claim 13 which further comprises a pharmaceutically acceptable carrier.

15. A pharmaceutical composition according to Claim 14 wherein said inhibitor has an EC_{50} value from about 0.1nanoM to about 10 microM.

16. A pharmaceutical composition according to Claim 13 which further comprises a therapeutically effective amount of a bisphosphonate active.

17. A pharmaceutical composition according to Claim 16 wherein said bisphosphonate active corresponds to the chemical structure



wherein n is an integer from 0 to 7 and wherein A and X are independently selected from the group consisting of H, OH, halogen, NH_2, SH, phenyl, C1-C30 alkyl, C3-C30 branched or cycloalkyl, C1-C30 substituted alkyl, C1-C10 alkyl substituted NH_2, C3-C10 branched or cycloalkyl substituted NH_2, C1-C10 dialkyl substituted NH_2, C1-C10 alkoxy, C1-C10 alkyl substituted thio, thiophenyl, halophenylthio, C1-C10 alkyl substituted phenyl, pyridyl, furanyl, pyrrolidinyl, imidazolyl, imidazopyridinyl, and benzyl; or A and X are taken together with the carbon atom or atoms to which they are attached to form a C3-C10 ring; and provided that when n is 0, A and X are

not selected from the group consisting of H and OH; and the pharmaceutically acceptable salts thereof.

18. A pharmaceutical composition according to Claim 16 wherein said bisphosphonate active is selected from the group consisting of alendronate, cimadronate, clodronate, tiludronate, etidronate, ibandronate, neridronate, olpandronate, risedronate, piridronate, pamidronate, zolendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

19. A pharmaceutical composition according to Claim 18 wherein said bisphosphonate active is alendronate, pharmaceutically acceptable salts thereof, and mixtures thereof.

20. A pharmaceutical composition according to Claim 19 wherein said bisphosphonate active is alendronate monosodium trihydrate.

21. A method for identifying a compound which inhibits apoptosis of cells of osteoblastic lineage comprising:

a). contacting a putative inhibitor of apoptosis of cells of osteoblastic lineage with a cell culture; and

b). determining the inhibition by comparing with a cell culture not contacted with said putative inhibitor.

INTERNATIONAL SEARCH REPORT

International application No.: PCT/US99/23755

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :A61K 31/19, 31/66, 31/35

US CL :514/102, 107, 108, 454, 573

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/102, 107, 108, 454, 573

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 4,782,082 A (KREUTNER et al.) 01 November 1988 see entire document.	1-21
A	US 5,366,965 A (STREIN) 22 November 1994 see entire document.	1-21
A,P see entire document	US 5,858,778 A (ALNEMRI et al.) 12 January 1999.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

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